Technical Manual

MSI Analysis System, Version 1.2

INSTRUCTIONS FOR USE OF PRODUCT MD1641

This Technical Manual has been revised. Please read it carefully.

- The MSI Analysis System, Version 1.2, incorporates improved manufacturing processes.
- A new matrix must be generated using one of the new matrices: PowerPlex® Matrix Standards 310 (Cat.# DG4640) or PowerPlex® Matrix Standards 3100/3130 (Cat.# DG4650).

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Part# TM255

MSI Analysis System, Version 1.2

Promega

All technical literature is available on the Internet at: www.promega.com/tbs/
Please visit the web site to verify that you are using the most current version of this Technical Manual.
Please contact Promega Technical Services if you have questions on use of this system.
E-mail: techserv@promega.com

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I. Description

I.A. The MSI Analysis System

The MSI Analysis System, Version 1.2[a–e], is a fluorescent PCR-based assay to detect microsatellite instability. Typically, MSI analysis involves comparison of allelic profiles of microsatellite markers generated by amplification of DNA from matching normal and tumor samples. Alleles that are present in the tumor sample but not found in the corresponding normal samples indicate MSI. The MSI Analysis System includes fluorescently labeled primers for co-amplification of seven markers including five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D) (Figure 1 and Table 1). The mononucleotide markers are used for MSI determination, and the pentanucleotide markers are used to detect potential sample mix-ups and/or contamination. Internal lane size standards (Figure 2) are added to the amplified samples to assure accurate sizing of alleles and adjust for run-to-run variation. The PCR products are separated by capillary electrophoresis (CE) using an ABI PRISM® 310 or 3100 or Applied Biosystems 3130 or 3130*xl* genetic analyzer, and the output data may be analyzed with the GeneMapper® software (Applied Biosystems) to determine MSI status of tumor samples. To simplify data analysis, we have created panel and bin files to allow automatic assignment of genotypes using GeneMapper® software. The proper panel and bin files for use with GeneMapper® software can be obtained from the Promega web site at: **www.promega.com/techserv/tools/msi/**

Table 1. The MSI Analysis System Locus Information.

Marker Name	GenBank® Number	Major Repeat Sequence	Size Range (bp)[1]	K562 Alleles (bp)[2]	Primer Dye
NR-21[3]	XM_033393	$(A)_{21}$	94–101	101	JOE
BAT-26	U41210	$(A)_{26}$	103–115	113	FL
BAT-25	L04143	$(A)_{25}$	114–124	122	JOE
NR-24[3]	X60152	$(A)_{24}$	130–133	130	TMR
MONO-27	AC007684	$(A)_{27}$	142–154	150	JOE
Penta C	AL138752	$(AAAAG)_{3–15}$	143–194	164, 174	TMR
Penta D	AC000014	$(AAAAG)_{2–17}$	135–201	168, 187	FL

[1]Amplicon size range is based on genotyping 300–500 individuals from three different racial groups (Caucasian-American, Asian-American and African-American). Rare alleles outside these size ranges may exist.

[2]Allele sizes determined using the ABI PRISM® 3100 genetic analyzer.

[3]See reference 1.

The mononucleotide repeat markers included in the MSI Analysis System were selected for high sensitivity and specificity to alterations in tumor samples with mismatch repair defects (1–3). These mononucleotide repeat markers are quasi-monomorphic; that is, almost all individuals are homozygous for the same common allele for a given marker (see Section XI.B for allele frequencies). Use of monomorphic markers simplifies data interpretation. The pentanucleotide repeat markers have been selected for their high level of polymorphism and low degree of MSI to uniquely identify samples, helping confirm that tumor and matching normal samples are from the same individual.

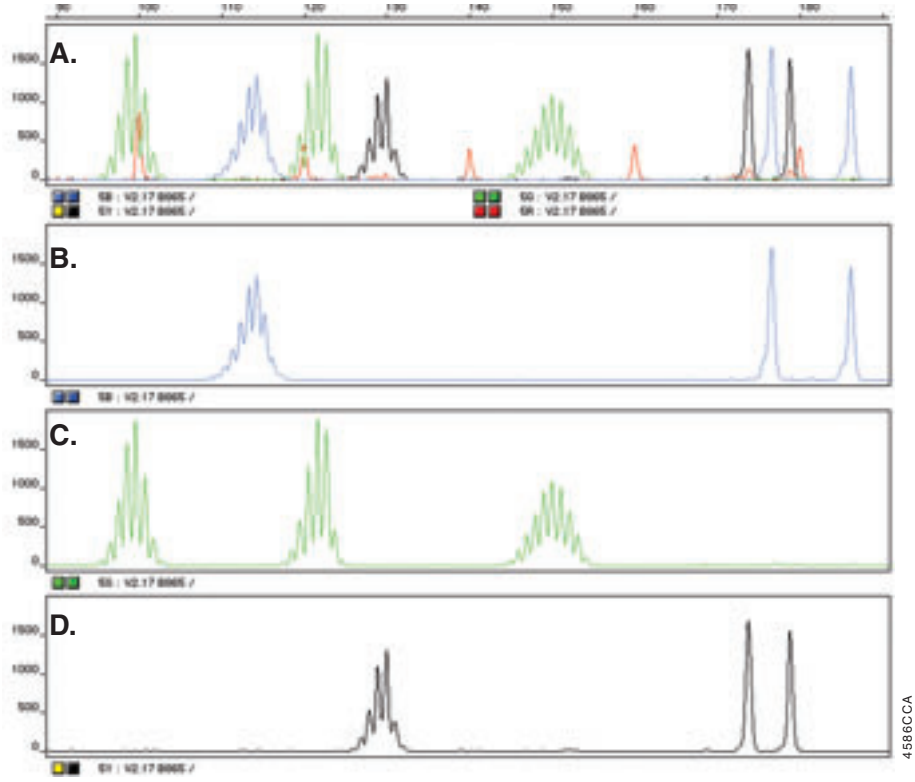

Figure 1. The MSI Analysis System, Version 1.2. A single normal genomic DNA template (1–2ng) was amplified using the MSI Analysis System, and the PCR products were analyzed using an ABI PRISM® 3100 genetic analyzer. **Panel A.** An electropherogram shows the five mononucleotide loci, two pentanucleotide loci and Internal Lane Standard 600 peaks. **Panel B.** An electropherogram showing peaks of the fluorescein-labeled loci, BAT-26 and Penta D. **Panel C.** An electropherogram showing the peaks of JOE-labeled loci, NR-21, BAT-25 and MONO-27. **Panel D.** An electropherogram showing the peaks of TMR-labeled loci, NR-24 and Penta C.

I.B. Microsatellite Instability Testing Overview

Microsatellites are short, tandemly repeated DNA sequences from 1–6 base pairs in length (4,5). These repeats are distributed throughout the human genome and often vary in length from one individual to another due to differences in the number of tandem repeats at each locus. Microsatellite markers can be used to detect a form of genomic instability called microsatellite instability (MSI) (6–9). MSI is a change in length of a microsatellite allele due to insertion or deletion of repeating units during DNA replication and failure of the DNA mismatch repair system to correct these errors. MSI analysis can be used as a screening method to identify samples for further characterization.

In 1997, a National Cancer Institute (NCI) workshop recommended a panel of five microsatellite markers to detect MSI consisting of two mononucleotide markers and three dinucleotide repeats (10). Samples with instability in two or more of these markers are defined as MSI-High (MSI-H), whereas those with one unstable marker are designated as MSI-Low (MSI-L). Samples with no detectable alterations are MSI-stable (MSS). Limitations in the original panel resulting from inclusion of dinucleotide repeats were addressed at a 2002 NCI workshop, and revised recommendations for MSI detection were proposed (11). It was recommended to substitute mononucleotide for dinucleotide repeat markers to improve sensitivity and specificity for detection of MSI.

I.C. The Internal Lane Standard 600

The Internal Lane Standard 600 (ILS 600) contains 22 DNA fragments of 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550 and 600 bases in length (Figure 2). Each fragment is labeled with carboxy-X-rhodamine (CXR) and is detected separately (as a fourth color) in the presence of MSI Analysis System-amplified material using the ABI PRISM® 310 or 3100 or Applied Biosystems 3130 or 3130*xl* genetic analyzer. The ILS 600 is designed for use in each CE injection to increase precision in analysis when using the MSI Analysis System. Protocols for preparation and use of this internal lane standard are provided in Sections V.B, VI.B and VII.B.

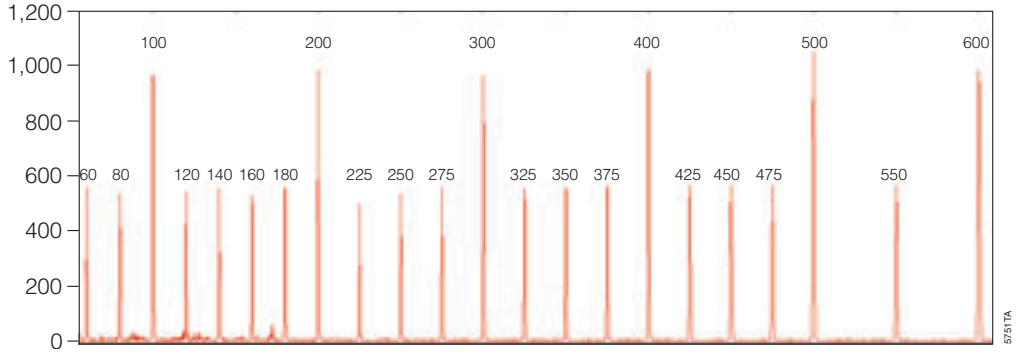

Figure 2. Internal Lane Standard 600 (ILS 600).

II. Product Components and Storage Conditions

Product	Size	Cat.#
MSI Analysis System, Version 1.2	100 reactions (50 reaction pairs)	MD1641

For In Vitro Research Use Only. Not For Use In Diagnostic Procedures.

Each system contains sufficient reagents to perform 100 reactions (50 reaction pairs). Includes:

Preamplification Components Box (Yellow Label)

- 1 × 100µl MSI 10X Primer Pair Mix
- 1 × 300µl Gold ST★R 10X Buffer
- 1 × 1.25ml Nuclease-Free Water
- 1 × 3µg K562 High Molecular Weight DNA (10ng/µl)

Postamplification Components Box (Magenta Label)

- 1 × 150µl Internal Lane Standard (ILS) 600
- 1 Protocol

Storage Conditions: Store all components at –20°C. The MSI 10X Primer Pair Mix and the Internal Lane Standard 600 are light-sensitive and must be stored in the dark.

Available Separately

Product	Size	Cat.#
PowerPlex® Matrix Standards 310	1 each	DG4640
PowerPlex® Matrix Standards 3100/3130	1 each	DG4650

Not for Medical Diagnostic Use.

III. DNA Extraction Methods

Obtaining sufficient high-quality DNA from formalin-fixed, paraffin-embedded tissues can be problematic. DNA is often degraded due to prolonged or unsuitable fixation of the tissue sample before embedding in paraffin. Therefore, we recommend using the MagneSil® Genomic, Fixed Tissue System (Cat.# MD1490) to extract DNA from paraffin-embedded tissues when using the MSI Analysis System. The MagneSil® Genomic, Fixed Tissue System provides a simple technique to prepare genomic DNA from formalin-fixed, paraffin-embedded tissue. After an overnight Proteinase K digestion, genomic DNA can be manually purified from formalin-fixed, paraffin-embedded thin tissue sections in less than an hour. Amplifiable genomic DNA can be isolated from 10µm thin sections without the use of organic solvents or centrifugation of the lysate prior to purification. Samples can be processed in the manual format using the MagneSphere® Technology Magnetic Separation Stand (twelve-position) (Cat.# Z5342). DNA extraction using the MagneSil® Genomic, Fixed Tissue System can also be automated. Please contact your local Promega Branch Office or Distributor (contact information available at: **www.promega.com**) or email: **techserv@promega.com** for more information.

IV. DNA Amplification Using the MSI Analysis System

Materials to Be Supplied by the User

- thermal cycler [GeneAmp® system 9600 or 9700 (Applied Biosystems)]
- microcentrifuge
- 0.2ml (thin-walled) microcentrifuge tubes, MicroAmp® reaction tube strips or MicroAmp® optical 96-well reaction plates (Applied Biosystems)
- 1.5ml amber-colored microcentrifuge tubes
- aerosol-resistant pipette tips
- AmpliTaq Gold® DNA polymerase (Applied Biosystems)

The MSI Analysis System is optimized to amplify 1–2ng of genomic DNA in a 10µl reaction volume using the protocols detailed below. However, optimization of input DNA amounts should be performed in individual laboratories to adjust for variations in yield and quality of DNA resulting from differences in samples and/or DNA isolation methods. Using excessive amounts of DNA template may result in peak heights exceeding the linear detection range of the CE instruments. Use of insufficient DNA template can result in low PCR yields, and peak heights may fall below detection limits (50RFU). Accurate quantitation of template DNA is highly recommended.

The MSI Analysis System is optimized for use with the GeneAmp® PCR system 9600 and 9700 thermal cyclers.

IV.A. Amplification Setup

Note: We strongly recommend using gloves and aerosol-resistant pipette tips to prevent cross-contamination. We recommend keeping all preamplification and postamplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup.

1. Thaw the Gold ST★R 10X Buffer and MSI Analysis System 10X Primer Pair Mix.
2. Mix these reagents by vortexing for 5–10 seconds before each use. A precipitate may form in the Gold ST★R Buffer. If this occurs, warm the buffer briefly at 37°C, then vortex until it is in solution.
3. To prepare the Amplification Mix, determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for losses during pipetting. This approach ensures that you will have enough PCR master mix for all samples. It also ensures that each reaction contains the same master mix.
4. Place one 0.2ml microcentrifuge tube for each reaction into a rack, and label appropriately. Alternatively use MicroAmp® optical 96-well reaction plates.

5. The following table shows the component volumes per sample when using a DNA template volume of 2µl in a 10µl reaction volume. If a different template volume is required, the water volume should be adjusted accordingly.

Amplification Mix for the MSI Analysis System

Component	Volume Per Sample
Nuclease-Free Water	5.85µl
Gold ST★R 10X Buffer	1.00µl
MSI Analysis System 10X Primer Pair Mix	1.00µl
AmpliTaq Gold® DNA polymerase (5u/µl)	0.15µl
total reaction volume	**8.00µl**

Note: Pipetting volumes smaller than 1µl is inaccurate. We recommend preparing enough Amplification Mix to avoid pipetting such small volumes.

6. Combine the final volumes of Nuclease-Free Water, Gold ST★R 10X Buffer, MSI Analysis System 10X Primer Pair Mix and AmpliTaq Gold® DNA polymerase in a sterile, 1.5ml amber tube. Mix gently.

7. Transfer 8µl of the Amplification Mix to the bottom of each reaction tube or well.

8. Pipet 2µl of the template DNA (1–2ng) for each sample into the bottom of the respective tube or well containing Amplification Mix. Mix by pipetting several times.

(!) Be sure that the template DNA is **well mixed** before transferring it into the bottom of the tube or well containing the Amplification Mix.

Note: Store DNA templates in nuclease-free water or TE^{-4} buffer [10mM Tris HCl (pH 8.0), 0.1mM EDTA]. If the template DNA is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA sample added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations depending on the source of the template DNA and the extraction procedure used.

9. For the positive amplification control, dilute the K562 Genomic DNA sample 1:10 to 1ng/µl in Nuclease-Free Water. Pipet 2ng of the diluted DNA into the bottom of the tube or well containing the Amplification Mix. Mix by pipetting several times.

10. For the negative amplification control, pipet Nuclease-Free Water (instead of template DNA) into a microcentrifuge reaction tube or well containing the Amplification Mix. Mix by pipetting several times.

IV.B. Amplification Thermal Cycling

1. Assemble the tubes or MicroAmp® optical 96-well reaction plates in a thermal cycler.
2. Select and run a recommended protocol for the GeneAmp® PCR system 9600 or the GeneAmp® PCR system 9700 provided below.

Protocol for the Perkin-Elmer GeneAmp® PCR System 9600 Thermal Cycler

Cycling profile:

95°C for 11 minutes, then:

96°C for 1 minute, then:

94°C for 30 seconds
ramp 68 seconds to 58°C, hold for 30 seconds
ramp 50 seconds to 70°C, hold for 1 minute
for **10 cycles**, then:

90°C for 30 seconds
ramp 60 seconds to 58°C, hold for 30 seconds
ramp 50 seconds to 70°C, hold for 1 minute
for **20 cycles**, then:

60°C for 30 minutes

4°C soak

Protocol for the Perkin-Elmer GeneAmp® PCR System 9700 Thermal Cycler

Note: Perkin-Elmer GeneAmp® PCR system 9700 thermal cycling conditions are in 9600 emulation mode and use the silver block.

Cycling profile:

95°C for 11 minutes, then:

96°C for 1 minute, then:

ramp 100% to 94°C for 30 seconds,
ramp 29% to 58°C for 30 seconds,
ramp 23% to 70°C for 1 minute,
for **10 cycles**, then:

ramp 100% to 90°C for 30 seconds,
ramp 29% to 58°C for 30 seconds,
ramp 23% to 70°C for 1 minute,
for **20 cycles**, then:

60°C for 30 minutes

4°C soak

3. After completion of the thermal cycling protocol, store the samples at –20°C, protected from light.

   Note: DNA quantity and quality affect PCR yield. Use the recommended DNA purification methods and accurate quantitation to minimize problems. Cycle number may be modified to adjust for variations in DNA amount but should not exceed 32 cycles.

V. Detection of Amplified Fragments Using the ABI PRISM® 310 Genetic Analyzer

Materials to Be Supplied by the User

- dry heating block, water bath, or thermal cycler
- 310 capillaries, 47cm × 50µm (Applied Biosystems)
- performance optimized polymer 4 (POP-4™) (Applied Biosystems)
- 10X genetic analyzer buffer with EDTA (Applied Biosystems)
- sample tubes and septa (Applied Biosystems)
- aerosol-resistant pipette tips
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)
- PowerPlex® Matrix Standards, 310 (Cat.# DG4640)
- crushed ice

Note: The quality of the formamide is critical. Use Hi-Di™ formamide with a conductivity less than 100µS/cm. Freeze formamide in aliquots at –20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause a breakdown of the formamide. Formamide with conductivity greater than 100µS/cm may contain ions that compete with DNA during injection. This results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

Caution: Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

V.A. Matrix Standardization

The PowerPlex® Matrix Standards, 310 (Cat.# DG4640), are required for matrix standardization on the ABI PRISM® 310 genetic analyzer. The PowerPlex® Matrix Standards, 3100/3130 (Cat.# DG4650), cannot be used to generate a matrix on the ABI PRISM® 310 genetic analyzer. For protocols and additional information on matrix standardization, see the *PowerPlex® Matrix Standards, 310 Technical Bulletin* #TBD021 (supplied with Cat.# DG4640) available upon request from Promega or online at: **www.promega.com/tbs/**

Proper generation of a matrix file is critical to evaluate multicolor systems with the ABI PRISM® 310 genetic analyzer. A matrix must be generated for each ABI PRISM® 310 genetic analyzer. For answers to questions about matrix creation send an e-mail message to: **techserv@promega.com**

V.B. Sample Preparation

The Internal Lane Standard 600 is included in each experiment to standardize the analysis of amplified samples and uses a fourth color.

1. Prepare a loading cocktail by combining and mixing the Internal Lane Standard 600 (ILS 600) and deionized formamide as follows:

   [(1.0µl ILS 600) + (24µl deionized formamide) × (# injections)]

2. Combine 25µl of the prepared loading cocktail and 1µl of amplified sample.

   Note: Instrument detection limits vary; therefore, injection time or the amount of product mixed with loading cocktail may need to be increased or decreased. If the peak heights are too high (i.e., greater than 3,000RFU), the samples can be diluted in Gold ST★R 1X Buffer before mixing with loading cocktail. This may result in uneven allele peak heights across loci. For best results, use less DNA template in the amplification reactions. The volume of ILS 600 can also be adjusted.

3. Denature the samples and ladder by heating at 95°C for 3 minutes and immediately chill on crushed ice for 3 minutes. Denature the samples just prior to loading.

4. Assemble the tubes in the appropriate autosampler tray (48- or 96-tube).

5. Place the autosampler tray in the instrument, and close the instrument doors.

V.C. Instrument Preparation

Refer to the ABI PRISM® genetic analyzer user's manual for instructions on cleaning the pump block, installing the capillary, calibrating the autosampler and adding polymer to the syringe.

1. Open the ABI PRISM® 310 collection software.

2. Prepare a GeneMapper® sample sheet as described in the ABI PRISM® 310 genetic analyzer user's manual. Enter the appropriate sample information in the sample info column.

3. Create a new GeneMapper® injection list. Select the appropriate sample sheet by using the pull-down menu.

4. Select the "GS STR POP4 (1ml) A" Module using the pull-down menu. Change the injection time to 2 seconds, and keep the settings for the remaining parameters as shown below:

Inj. Secs:	1–5
Inj. kV:	15.0
Run kV:	15.0
Run °C:	60
Run Time:	30

   Note: You may need to optimize injection time for individual instruments. Injection times of 1–5 seconds are recommended with 1–2ng of template DNA.

5. Select the appropriate matrix file (Section V.A).

6. To analyze the data automatically, select the auto-analyze check box and the appropriate analysis parameters and size standard. Refer to the ABI PRISM® 310 genetic analyzer user's manual for specific information on these options.

7. After loading the sample tray and closing the doors, select "Run" to start the capillary electrophoresis system.

8. Monitor the electrophoresis by observing the raw data and status windows. Each sample will take approximately 30 minutes for syringe pumping, sample injection and sample electrophoresis.

VI. Detection of Amplified Fragments Using the ABI PRISM® 3100 Genetic Analyzer with Data Collection Software, Version 1.01 or 1.1

Materials to Be Supplied by the User

- dry heating block, water bath, or thermal cycler
- crushed ice
- aerosol-resistant pipette tips
- 3100 capillary array, 36cm
- performance optimized polymer 4 (POP-4™) (Applied Biosystems)
- 10X genetic analyzer buffer with EDTA (Applied Biosystems)
- sample tubes and septa for the 3100 (Applied Biosystems)
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)
- PowerPlex® Matrix Standards, 3100 (Cat.# DG4650)

Note: The quality of the formamide is critical. Use Hi-Di™ formamide with a conductivity less than 100µS/cm. Freeze formamide in aliquots at –20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause a breakdown of the formamide. Formamide with conductivity greater than 100µS/cm may contain ions that compete with DNA during injection. This results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

Caution: Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

VI.A. Spectral Calibration

The PowerPlex® Matrix Standards, 3100/3130 (Cat.# DG4650) are required for spectral calibration on the ABI PRISM® 3100 genetic analyzer. The PowerPlex® Matrix Standards, 310 (Cat.# DG4640) cannot be used to generate a matrix on the ABI PRISM® 3100 genetic analyzer. For protocols and additional information on spectral calibration, see the *PowerPlex® Matrix Standards, 3100/3130 Technical Bulletin* #TBD022 (supplied with Cat.# DG4650) available upon request from Promega or online at: **www.promega.com/tbs/**

Proper spectral calibration is critical to evaluate multicolor systems with the ABI PRISM® 3100 genetic analyzer. Spectral calibration must be performed for each ABI PRISM® 3100 genetic analyzer. For answers to questions about matrix creation send an e-mail to: **techserv@promega.com**

VI.B. Sample Preparation

The Internal Lane Standard 600 is included in each experiment to standardize analysis of amplified samples and uses a fourth color.

1. Prepare a loading cocktail by combining and mixing the Internal Lane Standard 600 (ILS 600) and deionized formamide as follows:

   [(0.5µl ILS 600) × (# injections)] + [(9.5µl deionized formamide) × (# injections)]

   Note: The volume of internal lane standard used in the loading cocktail can also be increased or decreased to adjust the intensity of the size standard peaks. The optimal peak height for the 100-base fragment of the internal lane standard is 500–1,000RFU. If the peak heights are too low, we recommend altering the formamide/internal lane standard mix to contain 1.0µl of ILS 600 and 9.0µl of Hi-Di™ formamide. If the peak heights are too high, we recommend altering the loading cocktail to contain 0.25µl of ILS 600 and 9.75µl of formamide.

2. Vortex for 10–15 seconds.

3. Pipet 10µl of formamide/internal lane standard mix into each well.

4. Add 1µl of amplified sample. Cover wells with appropriate septa.

   Note: Instrument detection limits vary; therefore, the injection time or the amount of product mixed with loading cocktail may need to be increased or decreased. If peak heights are too high (>4,000–6,000RFU), the samples can be diluted in Gold ST★R 1X Buffer before mixing with loading cocktail.

5. Centrifuge plate briefly to remove air bubbles from the wells if necessary.

6. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice for 3 minutes. Denature the samples just prior to loading the instrument.

VI.C. Instrument Preparation

Refer to the ABI PRISM® 3100 genetic analyzer user's manual for instructions on cleaning the pump blocks, installing the capillary array, performing a spatial calibration and adding polymer to the reserve syringe.

1. Open the ABI PRISM® 3100 data collection software.

2. Open a new plate record. Name the plate, and select "GeneScan". Select the plate size (96-well or 384-well). Select "Finish".

3. Complete the plate record spreadsheet for the well you have loaded. Enter appropriate information into the "Sample Name" column (Figure 3).

4. In the "Project Name" column, select "3100_Project1" from the pull-down menu.

5. In the "Dye Set" column, select "F" from the pull-down menu.

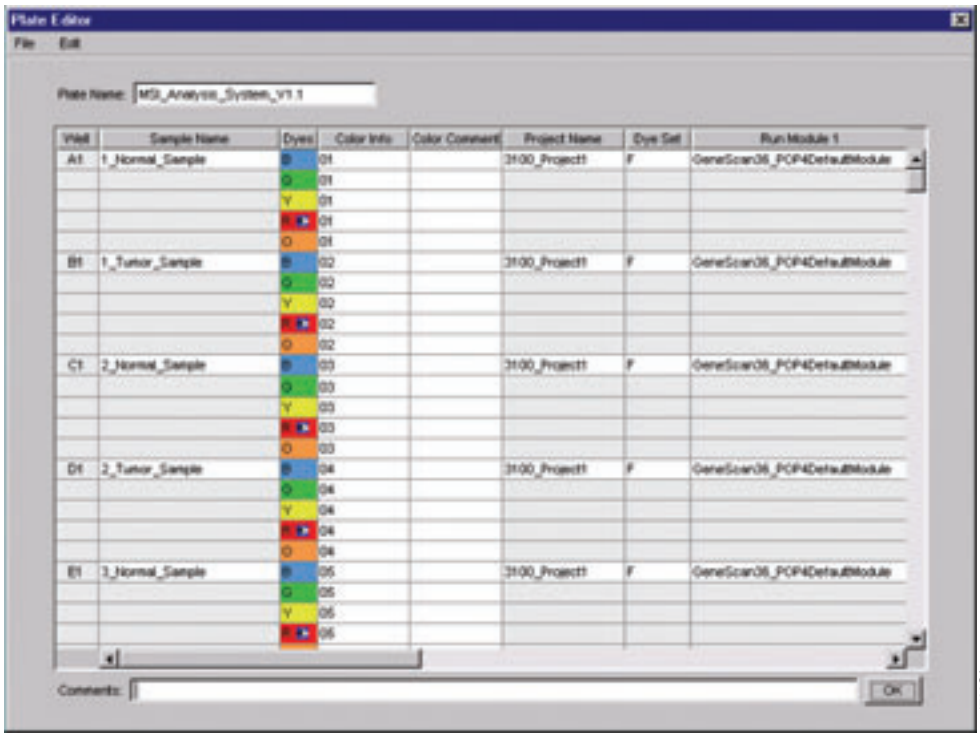

Figure 3. The Plate Editor.

6. In the "Run Module 1" column, select "GeneScan36_POP4DefaultModule" from the pull-down menu.

7. To collect the data without autoanalyzing, select "No Selection" in the "Analysis Module 1" column. Analysis parameters can be applied after data collection and during data analysis using the GeneMapper® analysis software.

   To analyze the data during data collection, an appropriate analysis module must be selected in the "Analysis Module 1" column. Refer to the ABI PRISM® 3100 genetic analyzer user's manual for specific instructions on creating analysis modules.

8. Select "OK." This new plate record will appear in the pending plate records table on the plate setup page of the collection software.

9. Place the samples in instrument, and close the instrument doors.

10. In the pending plate records table, click once on the name of the plate record you just created.

11. Once the plate record is highlighted, click the plate graphic corresponding to the plate on the autosampler that contains your amplified samples.

12. When the plate record is linked to the plate, the plate graphic will change from yellow to green, the plate record moves from the pending plate records table to the linked plate records table, and the "Run Instrument" button becomes enabled (Figure 4).

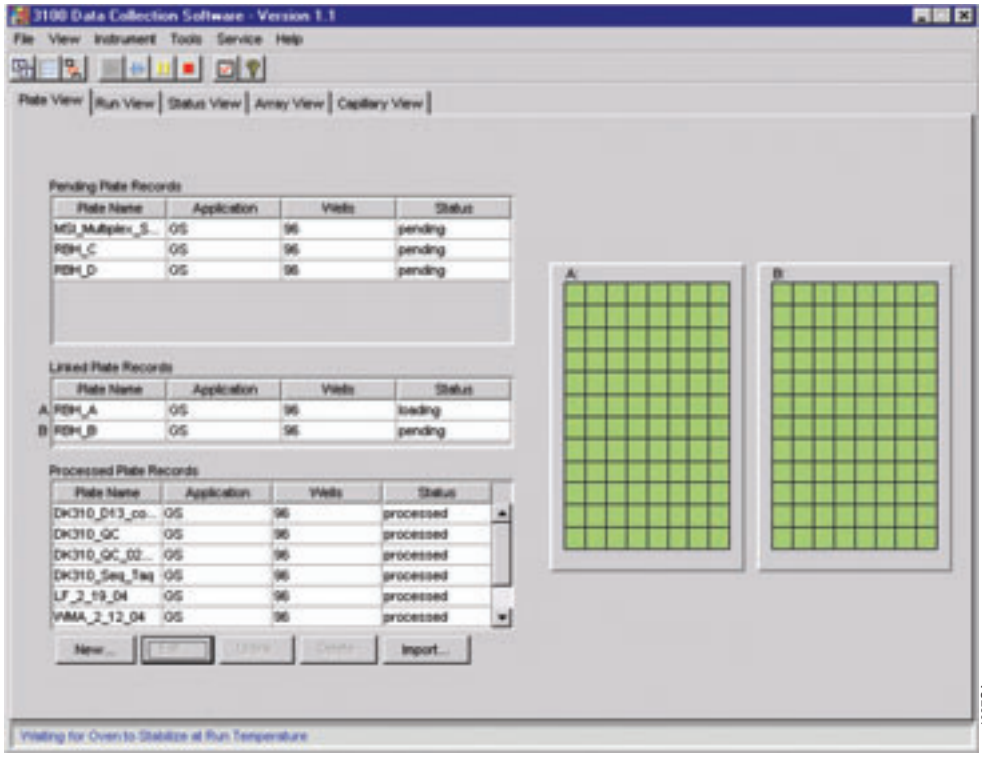

Figure 4. The Plate View tab.

13. Select "Run Instrument" on the tool bar to start the sample run.

14. Monitor electrophoresis by observing the run, status, array and capillary views windows in the collection software. Each run (16 samples/ capillaries) will take approximately 45 minutes.

VII. Detection of Amplified Fragments Using the ABI PRISM® 3100 or 3100-*Avant* Genetic Analyzer with Data Collection Software, Version 2.0, and the Applied Biosystems 3130 or 3130*xl* Genetic Analyzer

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice
- aerosol-resistant pipette tips
- 3100 or 3130 capillary array, 36cm
- performance optimized polymer 4 (POP-4™) (Applied Biosystems)
- 10X genetic analyzer buffer with EDTA (Applied Biosystems)
- MicroAmp® optical 96-well plate and septa (Applied Biosystems)
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)
- PowerPlex® Matrix Standards, 3100/3130 (Cat.# DG4650)

The quality of the formamide is critical. Use Hi-Di™ formamide with a conductivity less than 100µS/cm. Freeze formamide in aliquots at –20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause a breakdown of the formamide. Formamide with a conductivity greater than 100µS/cm may contain ions that compete with DNA during injection. This results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

Caution: Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

VII.A. Spectral Calibration

The PowerPlex® Matrix Standards, 3100/3130 (Cat.# DG4650) are required for spectral calibration on the ABI PRISM® 3100 genetic analyzer. The PowerPlex® Matrix Standards, 310 (Cat.# DG4640) cannot be used to generate a matrix on the ABI PRISM® 3100 genetic analyzer. For protocols and additional information on spectral calibration, see the *PowerPlex® Matrix Standards, 3100/3130 Technical Bulletin* #TBD022 (supplied with Cat.# DG4650) available upon request from Promega or online at: **www.promega.com/tbs/**

Proper spectral calibration is critical to evaluate multicolor systems with the ABI PRISM® 3100 genetic analyzer. Spectral calibration must be performed for each ABI PRISM® 3100 genetic analyzer. For answers to questions about matrix creation send an e-mail to: **techserv@promega.com**

VII.B. Sample Preparation

The Internal Lane Standard 600 is included in each experiment to standardize analysis of amplified samples and uses a fourth color.

1. Prepare a loading cocktail by combining and mixing the internal lane standard and Hi-Di™ formamide as follows:

   [(0.5µl ILS 600) × (# injections)] + [(9.5µl Hi-Di™ formamide) × (# injections)]

   Note: The volume of internal lane standard used in the loading cocktail can also be increased or decreased to adjust the intensity of the size standard peaks. The optimal peak height for the 100-base fragment of the internal lane standard is 500–1,000RFU. If the peak heights are too low, we recommend altering the formamide/internal lane standard mix to contain 1.0µl of ILS 600 and 9.0µl of Hi-Di™ formamide. If the peak heights are too high, we recommend altering the loading cocktail to contain 0.25µl of ILS 600 and 9.75µl of formamide.

2. Mix for 10–15 seconds using a vortex mixer.

3. Pipet 10µl of formamide/internal lane standard mix into each well.

4. Add 1µl of amplified sample. Cover wells with appropriate septa.

   Note: Instrument detection limits vary; therefore, injection time or the amount of product mixed with loading cocktail may need to be increased or decreased. Use the "Module Manager" in the data collection software to modify the injection time or voltage in the run module. If the peak heights are higher than desired, the samples can be diluted in Gold ST★R 1X Buffer before mixing with loading cocktail. This may result in uneven allele peak heights across loci. For best results, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program by 2–4 cycles to achieve the desired signal intensity.

5. Centrifuge plate briefly to remove air bubbles from the wells if necessary.

6. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice for 3 minutes. Denature the samples just prior to loading the instrument.

VII.C. Instrument Preparation

Refer to the instrument users' manual for instructions on cleaning, installing the capillary array, performing a spatial calibration and adding polymer.

Analyze the samples as described in the user's manual for the ABI PRISM® 3100 or 3100-*Avant* genetic analyzer with data collection software, version 2.0, and Applied Biosystems 3130 or 3130*xl* genetic analyzer with the following exceptions.

1. In the Module Manager, select "New". Select "Regular" in the Type drop-down list, and select "HIDFragmentAnalysis36_POP4" in the Template drop-down list. Confirm that the injection time is 5 seconds and the injection voltage is 3kV. Lengthen the run time to 2,000 seconds. Give a descriptive name to your run module, and select "OK".

   Note: Sensitivities of instruments may vary. The injection time and voltage may be adjusted in the Module Manager. A suggested range for the injection time is 3–22 seconds and for the injection voltage is 1–3kV.

2. In the Protocol Manager, select "New". Type a name for your protocol. Select "Regular" in the Type drop-down list, and select the run module you created in the previous step in the Run Module drop-down list. Lastly, select "F" in the Dye-Set drop-down list. Select "OK.

3. In the Plate Manager, create a new plate record as described in the instrument user's manual. In the dialog box that appears, select "GeneMapper—Generic" in the Application drop-down list, and select the appropriate plate type (96-well). Add entries in the owner and operator windows, and select "OK".

   Note: If autoanalysis of sample data is desired, refer to the instrument user's manual for instructions.

4. In the GeneMapper® plate record, enter sample names in the appropriate cells. Scroll to the right. In the "Results group 1" column, select the desired results group. In the "Instrument Protocol 1" column, select the protocol you created in Step 2. Be sure this information is present for each row that contains a sample name. Select "OK".

   Notes:

   1. To create a new results group, select "New" in the drop-down menu in the results group column. Select the General tab, and enter a name. Select the Analysis tab, and select "GeneMapper—Generic" in the Analysis type drop-down list.
   2. It is helpful to use names with similar initial characters when naming matched tumor and normal samples so that these samples remain together when analysis results are sorted.

5. Place samples in the instrument, and close the instrument doors.

6. In the spectral viewer, confirm that dye set F is active, and set the correct active calibration for dye set F.

7. In the run scheduler, locate the plate record that you just created in Steps 3 and 4, and click once on the name to highlight it.

8. Once the plate record is highlighted, click the plate graphic that corresponds to the plate on the autosampler that contains your amplified samples.

9. When the plate record is linked to the plate, the plate graphic will change from yellow to green, and the green Run Instrument arrow becomes enabled.

VII.C. Instrument Preparation (continued)

10. Click on the green Run Instrument arrow on the toolbar to start the sample run.
11. Monitor electrophoresis by observing the run, view, array or capillaries viewer window in the data collection software. Each injection will take approximately 45 minutes.

VIII. Data Analysis

VIII.A. MSI Analysis Overview

Detection of MSI is based on comparison of allelic profiles generated from amplification of matching normal and tumor DNA. The appearance of novel alleles in the tumor DNA indicates microsatellite instability (Figure 5). The MSI Analysis System allows the co-amplification and detection of a panel of microsatellite markers that have been shown to be sensitive and specific for detection of MSI-H tumors with mismatch repair deficiencies (2,3). Specific PCR product sizes and the respective fluorescent dyes used for labeling of each microsatellite marker are described in Table 1.

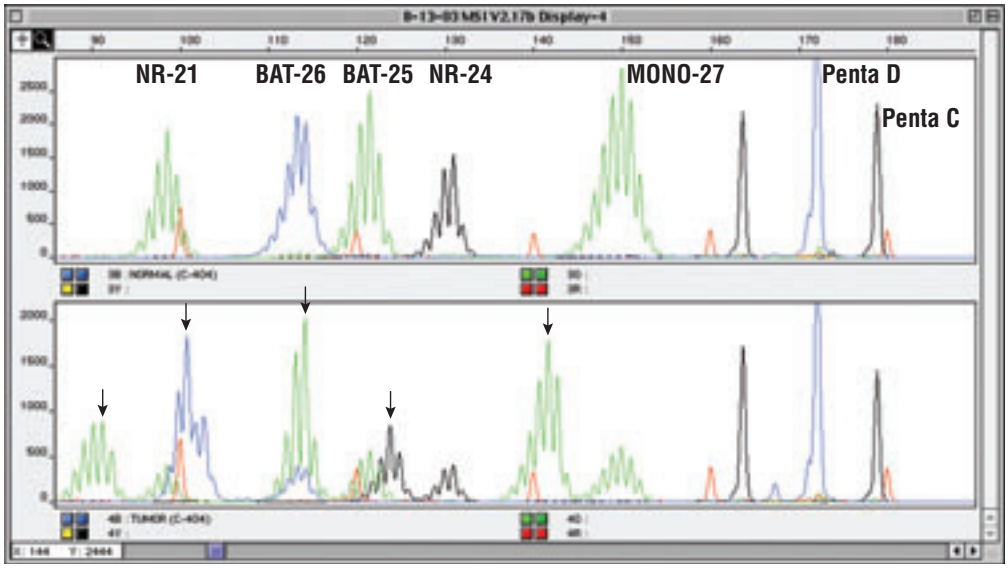

Figure 5. Microsatellite instability assays using the MSI Analysis System. Matching normal samples (top panel) and MSI-positive colon tumor samples (bottom panel) were analyzed using the MSI Analysis System. Two nanograms of genomic DNA were amplified and analyzed using an ABI PRISM® 3100 genetic analyzer, and the allelic patterns of the normal and tumor samples are shown. The presence of new alleles in the tumor sample (see arrows) that were not present in the normal sample indicates MSI. PCR product sizes and respective fluorescent dyes for loci contained in the MSI Analysis System are provided in Table 1.

The Bethesda guidelines (10,11) recommend that tumor samples in which ≥40% of microsatellite markers are altered (≥2 altered markers out of 5) be classified as MSI-H. Tumor samples with less than 40% mononucleotide repeat markers altered may be classified as nonMSI-H. However, the occurrence of even one altered mononucleotide marker in mismatch repair-proficient tumors is uncommon, and repeating the MSI assay or performing additional methods of analysis may be necessary to accurately classify these samples (3). Pentanucleotide markers are not intended for use in MSI classification.

Amplification of microsatellite markers will yield one or two major allele peaks, depending upon whether the individual is homozygous (one) or heterozygous (two) for that marker. In addition, amplification of microsatellite markers often generates artifact peaks called stutter (12). Electropherograms of mononucleotide markers show a number of less intense stutter peaks at 1bp intervals from the most prominent or true allele peak (Figure 6). A shift in allele size of 3bp or more in the tumor samples compared to matching normal samples is usually scored as MSI-positive for that marker. The mononucleotide markers included in the MSI Analysis System are nearly monomorphic so that most individuals are homozygous for the same common allele for a given marker (see Section XI.B for allele frequencies).

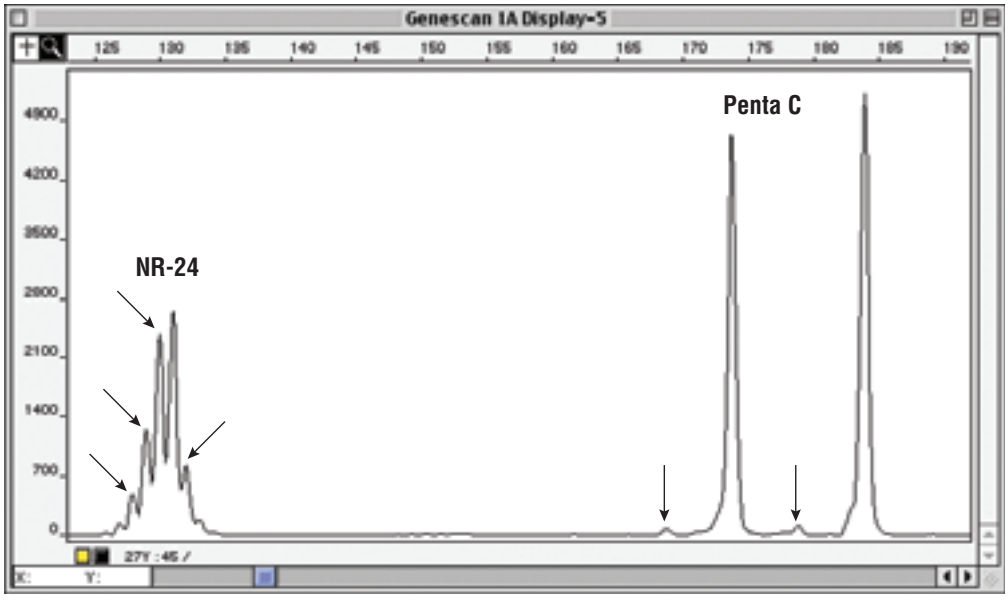

Figure 6. Stutter artifact peaks. Stutter peaks (arrows) generated by polymerase slippage during PCR amplification of short tandem repeats are clearly visible at the mononucleotide marker NR-24 (left) at 1bp intervals from the true or tallest allele peak. Much smaller stutter peaks can also be seen at the Penta C marker, 5bp from true allele peaks.

Two polymorphic pentanucleotide repeat markers, Penta C and Penta D, are included in the MSI Analysis System to help confirm that tumor and matching normal samples are from the same individual (13,14). Alleles found in the normal sample should also be present in the tumor if the tumor is from the same individual. MSI has been observed in 14% of MSI-H tumors in Penta C and 35% in Penta D, so additional pentanucleotide alleles may also be present in some MSI-H tumor samples (2). A matching probability between 1:10 and 1:16 was calculated for Penta C, and between 1:18 to 1:33 for Penta D based upon allele frequency data (13,14). Matching probability is the average number of randomly selected people you would have to survey before you would find two with the same allelic pattern. Using both pentanucleotide markers should allow detection of sample mix-ups over 99% of the time.

For data analysis, the GeneMapper® analysis software from Applied Biosystems is required. The software allows manual and automated analysis of the raw data and generates electropherograms with accompanying data tables displaying PCR fragment lengths (in base pairs) and quantitation of peak heights (in RFU). GeneMapper® results should be reviewed to eliminate artifact peaks caused by bleedthrough, stutter or CE spikes (Figures 6 and 7). Refer to the GeneMapper® analysis user's manual for detailed instructions on fragment analysis and genotyping. To simplify data analysis, we have created panel and bin files to allow automatic assignment of genotypes using GeneMapper® software. The proper panel and bin files for use with GeneMapper® software can be obtained from the Promega web site at: **www.promega.com/techserv/tools/msi/**

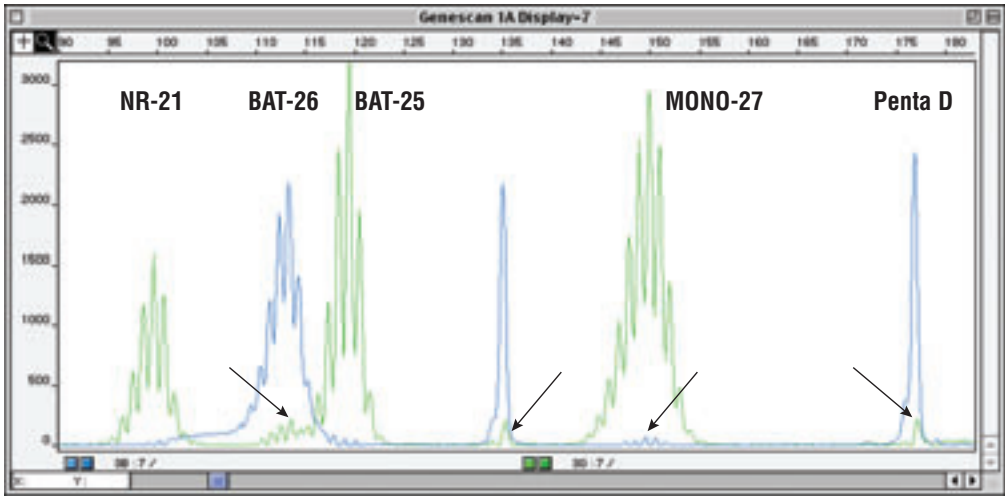

Figure 7. Bleedthrough or pull-up peaks. Smaller peaks (arrows) directly under much higher peaks in another color channel are called bleedthrough or pull-up peaks. Bleedthrough can occur when peak heights are excessive or if a poor or incorrect matrix has been applied to the samples.

VIII.B. Importing Panel and Bin Files for GeneMapper® Software, Version 4.0.

To facilitate analysis of data generated with the MSI Analysis System, Version 1.2, we have created panel and bin files to allow automatic assignment of genotypes using GeneMapper® software, version 4.0. For GeneMapper® software, version 3.5 or 3.7, we recommend upgrading to version 4.0. Version 4.0 has been improved to allow analysis of the mononucleotide and pentanucleotide repeats amplified by the MSI Analysis System.

Panel and bin files for the MSI Analysis System, Version 1.2, can be downloaded from the Promega web site at: **www.promega.com/techserv/tools/msi/**

1. Open the GeneMapper® software, version 4.0.
2. Select "Tools", then "Panel Manager".
3. Highlight the Panel Manager icon in the upper left tile (navigation pane).
4. From the menu, select "File", then "Import Panels".
5. Navigate to the file location where the panel and bin files have been saved on your computer. Select the file "Promega_Panels_MSI_GM4.0.0.txt", then "Import".
6. In the navigation pane, highlight the "Promega MSI" folder that you just imported.
7. Select "File", then "Import Bin Set".
8. Navigate to the file location where the panel and bin files have been saved on your computer. Select the file, "Promega_Bins_MSI_GM4.0.0.txt", then "Import".
9. At the bottom of the Panel Manager window, select "Apply", then "OK". The panel manager window will close automatically.

VIII.C. Creating an Analysis Method Using GeneMapper® Software, Version 4.0

1. Select "Tools", then "GeneMapper Manager".
2. Select the Analysis Methods tab.
3. Select "New", and a new analysis method dialog box will open.
4. Select "Microsatellite". Select "OK".
5. Enter a descriptive name for the analysis method, such as "Promega MSI".
6. Select the Allele tab.
7. Select the bin set corresponding to the Promega MSI System, "Promega_MSI".
8. Ensure that the "Use marker-specific stutter ratio if available" box is checked.

9. Enter the values shown in Figure 8 for proper filtering of peaks when using the MSI Analysis System. These filter settings will allow the user to display plotted data with the highest RFU peak labeled in each set of amplified fragments within each mononucleotide marker range. These settings can be changed if an alternative labeling strategy is desired. For an explanation of the proper usage and effect of these settings, refer to your GeneMapper® software version 4.0 documentation.

Figure 8. The Analysis Method Editor. The PlusA ratio setting for Mono repeats is 0.9999.

10. Select the Peak Detector tab. We recommend the settings shown in Figure 9. Peak Amplitude Thresholds should be determined by the individual laboratory based on desired sample peak intensity range, dynamic range of the instrument, and noise or background signal that is observed in analyzed data. Peak Amplitude Thresholds of between 50 and 200RFU are commonly used.

11. Select "OK" to save your settings.

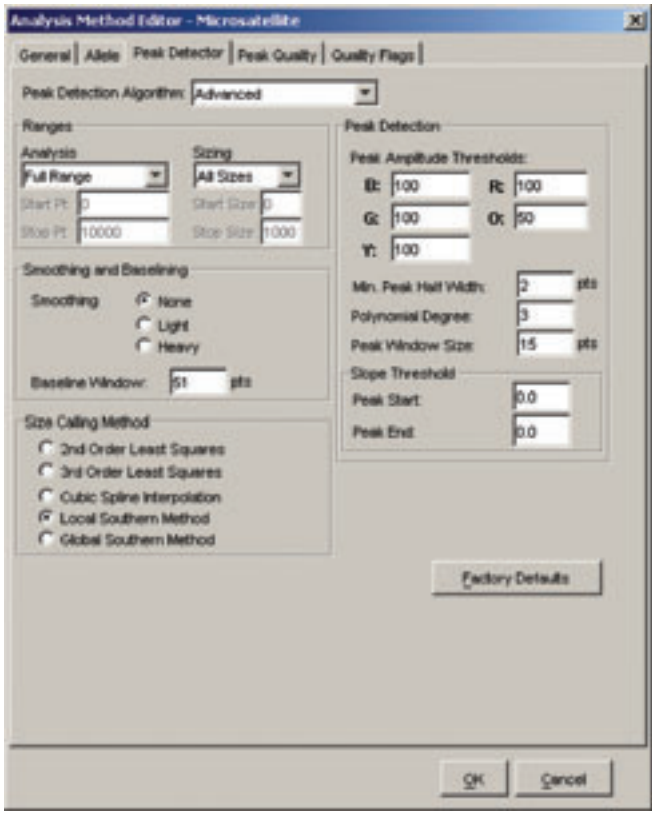

Figure 9. The recommended settings for the Peak Detector tab.

VIII.D. Creating a Size Standard

1. Select "Tools", then "GeneMapper Manager".
2. Select the Size Standard tab.
3. Select "New".
4. Select "Basic or Advanced" (Figure 9). The type of analysis method selected must match the peak detection algorithm selected on the peak detector tab for the analysis method. Select "OK".
5. Enter a detailed name, such as "ILS 600", in the Size Standard Editor. Make sure that red is selected as the color for the size standard dye.
6. Enter the sizes of the internal lane standard fragments into the table.
7. Select "OK".

VIII.E. Processing Data

1. In the File menu, select "New Project", then "Microsatellite".
2. Import sample files into a new project using the "Add Samples to Project" selection under the File menu.
3. In the Sample Type column, use the drop-down menu to select "Sample", "Positive Control" or "Negative Control" as appropriate.
4. In the Analysis Method column, select the analysis method created in Section VIII.D.
5. In the Panel column, select "Promega MSI" from the Promega MSI folder. This is the panel set that was imported in Section VIII.C.
6. In the Size Standard column, select the size standard that was created in Section VIII.E.
7. If analyzing data from an ABI PRISM® 310 genetic analyzer, ensure that the appropriate matrix file is selected in the Matrix column.
8. Select "Analyze" (green arrow button) to start the data analysis.

VIII.F. Reviewing the Size Standard

When correctly labeled, the ILS 600 should give a Sizing Quality score near 1.0. Problems with ILS analysis (extra peaks, mislabeling, etc.) will generate a Sizing Quality score of between 0.0 and 1.0 and may result in sample analysis failure. If analysis fails but the fragments have been assigned correctly, the Sizing Quality can be overridden by selecting "Override SQ". The samples can then be reanalyzed. For more information refer to Section IX or your GeneMapper® software, version 4.0, documentation.

1. Select all samples in the Samples tab by selecting "Edit", then "Select All".
2. Open the Size Match Editor by selecting "Analysis", then "Size Match Editor".
3. View the Size Matches tab to review the Size Quality score, size standard peaks and size standard peak labels for each sample.
4. Determine if all peaks in the size standard are present and labeled correctly for the Promega Internal Lane Standard (ILS) 600 as shown in Figure 2.

VIII.G. Reviewing Analyzed Sample Data

The GeneMapper® software, version 4.0, provides a variety of options for sample data display. The Promega MSI Analysis System panel and bin download package includes an informational document and an example plot settings file, which illustrates one option for review of sample data. For additional information on viewing sample plots please refer to your GeneMapper® software version 4.0 documentation.

IX. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Weak fluorescent signal for allele peaks	Insufficient template DNA. Make sure DNA is accurately quantitated, and use the recommended amount of template DNA.
	Degraded DNA. Prepare new genomic DNA.
	Impure DNA template. Impurities in DNA preparation may inhibit PCR. Clean up DNA or prepare new genomic DNA.
	Poor-quality DNA. Improper or prolonged fixation of paraffin-embedded samples can result in low DNA yields and poor-quality DNA. Repeat DNA preparation, or use the MagneSil® Genomic, Fixed Tissue System (Cat.# MD1490).
	Poor capillary electrophoresis injection (ILS 600 peaks also affected). Re-inject sample. Check instrument manual for instructions.
	Poor-quality formamide. Use high-quality formamide with conductivity of <100µS/cm.
	High salt concentration or altered pH. DNA volume should not exceed 20% of the total reaction volume. Carryover from DNA sample of K^+, Na^+, Mg^{2+} or EDTA can have a deleterious effect on PCR. Changes in pH may also affect PCR.
	Thermal cycler or tube problems. Confirm PCR program is correct. Use recommended thermal cycler and tubes. Calibration of heat block may be required.
	Samples not properly denatured before loading. Heat-denature samples for recommended time and cool on crushed ice immediately prior to loading the capillary.
	Amplification reaction components not added to the bottom of the tube or well. Gently tap the tube or plate to move the drop to bottom, or centrifuge briefly.
Excessive fluorescent signal for allele peaks	Too much template DNA. • Make sure DNA is accurately quantitated, and use 1–2ng in PCR. • Dilute PCR product 1:5 to 1:10 in sterile deionized water or 1X Gold ST★R Buffer prior to preparing loading solution.

IX. Troubleshooting (continued)

Symptoms	Causes and Comments
Extra peaks visible in one or all dye colors	Microsatellite artifacts (i.e., stutter). PCR amplification of microsatellite loci generates artifacts that appear as smaller peaks 1bp above or below the prominent mononucleotide repeat allele or 5bp below a pentanucleotide repeat allele. Stutter band peak heights will be higher if too much template DNA is used.
	Excess amount of DNA. One to two nanograms of DNA are recommended. Amplification of >2ng DNA template may result in a higher number of stutter bands. Use less DNA template, or reduce the number of cycles in the amplification program by 2 or more cycles.
	Pull-up or bleedthrough. Pull-up can occur when peak heights are excessive or if a poor or incorrect matrix has been applied to the samples. Increase the peak amplitude threshold (i.e., 150–200RFU) in the analysis method and reanalyze. If problems persist, generate a new matrix or perform a new spectral calibration, and apply to samples.
	Samples not properly denatured prior to loading. Heat-denature the samples for the recommended time and cool on crushed ice immediately prior to loading the capillary.
	CE-related artifacts (contaminants). Contaminants in the water used both on the ABI PRISM® 310 genetic analyzer and to dilute the 10X genetic analyzer buffer may generate peaks in the blue and green dye colors. Use autoclaved water, change vials and wash the buffer reservoir.
	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly.
Preferential amplification of smaller loci	DNA is cross-linked in sample. DNA prepared from paraffin-embedded samples often contain DNA that is cross-linked with other DNA or protein molecules, preventing amplification of longer DNA fragments. Use the MagneSil® Genomic, Fixed Tissue System to purify DNA.
	Degraded DNA. DNA template is degraded into smaller fragments with the larger loci showing diminished yield.
	Insufficient template DNA. Use the recommended amount of template DNA.
	Excess amounts of DNA. We recommend 1–2ng of DNA. Amplification of >2ng DNA template may result in an imbalance in yields with the smaller loci showing more amplification product than the larger loci. Use less DNA template, or reduce the number of cycles in the amplification program by 2 or more cycles.

X. References

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XI. Appendix

XI.A. Related Products

Product	Size	Cat.#
MagneSil® Genomic, Fixed Tissue System*	100 samples	MD1490
ART® 20P Pipet Tip, 20µl	960/pk	DY1071
ART® 100 Pipet Tip, 100µl	960/pk	DY1101
ART® 100E Pipet Tip, 100µl	960/pk	DY1111
ART® 200 Pipet Tip, 200µl	960/pk	DY1121
ART® 1000E Pipet Tip, 1,000µl	800/pk	DY1131

*For Laboratory Use.

XI.B. Allele Frequencies for Mononucleotide Repeat Loci

BAT-25			
Size (bp)	Caucasian	African	Asian
113	1	1	0
114	0	1	0
115	0	12	0
116	0	7	0
117	0	1	0
118	0	8	0
119	0	14	0
120	7	111	4
121	108	103	93
122	58	12	30
123	8	5	0
124	0	0	0
n =	**182**	**274**	**127**

NR-24			
Size (bp)	Caucasian	African	Asian
130	60	197	49
131	113	34	77
132	0	0	0
133	0	2	2
n =	**173**	**233**	**128**

NR-21			
Size (bp)	Caucasian	African	Asian
94	1	2	8
95	1	0	0
96	0	3	0
97	0	60	0
98	27	100	14
99	134	62	89
100	14	9	23
101	1	0	1
n =	**178**	**236**	**135**

BAT-26			
Size (bp)	Caucasian	African	Asian
103	0	23	0
104	0	0	0
105	0	0	0
106	0	2	1
107	1	0	0
108	0	0	0
109	0	0	0
110	0	0	0
111	0	4	0
112	14	38	3
113	140	131	115
114	22	57	8
115	0	0	0
n =	**177**	**255**	**127**

MONO-27			
Size (bp)	Caucasian	African	Asian
142	0	1	0
143	0	0	0
144	0	0	0
145	0	0	0
146	0	0	0
147	0	0	0
148	0	3	0
149	59	88	42
150	107	141	83
151	5	4	1
152	0	1	0
153	0	0	0
154	0	1	0
155	0	0	1
n =	**172**	**239**	**127**

[a]U.S. Pat. Nos. 6,844,152 and 7,202,031 and Australian Pat. No. 2001290868 have been issued to Promega Corporation for detection of microsatellite instability and its use in diagnosing tumors. Other patents are pending.

[b]U.S. Pat. Nos. 6,238,863 and 6,767,703 and Korean Pat. No. 691195 have been issued to Promega Corporation for materials and methods for identifying and analyzing intermediate tandem repeat DNA markers. Other patents are pending.

[c]Licensed under U.S. Pat. Nos. 6,566,053 and 7,090,978.

[d]This product is designed and sold for use in the multiplex PCR process covered by U.S. Pat. No. 5,582,989, Canadian Pat. No. 1,339,731 and Australian Pat. No. 634175. A limited license has been granted under the patent to use only this amount of the product to practice the multiplex PCR process and is conveyed to the purchaser by the purchase of this product.

[e]STR loci are the subject of U.S. Pat. No. RE 37,984, German Pat. No. DE 38 34 636 C2 and other patents issued to the Max-Planck-Gesellschaft zur Förderung der Wissenschaften, e.V., Germany. The development and use of STR loci are covered by U.S. Pat. No. 5,364,759, Australian Pat. No. 670231 and other pending patents assigned to Baylor College of Medicine, Houston, Texas.

Patents for the foundational PCR process, European Pat. Nos. 201,184 and 200,362, expired on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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